CHLOROPLAST DNA VARIABILITY AMONG PROVENANCES OF *Juniperus virginiana* AND *Juniperus scopulorum*

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Juniperus virginiana and Juniperus scopulorum

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INTRODUCTION

Juniperus virginiana and J. scopulorum are planted extensively in the Great Plains region of North America for windbreak, shelterbelt and conservation purposes. For example, in 1982 and 1983, over 2.7 million seedlings of both species were distributed through the Clarke-McNary Tree Distribution Program in the Great Plains region (Forestry Committee, Great Plains Agricultural Council 1984). Juniperus virginiana and J. scopulorum accounted for 84-percent of all conifer seedlings (13 species) and 37-percent of all conifer and hardwood seedlings combined (not including shrub species) distributed through this program in both 1982 and 1983. However, very little is known regarding the genetics and physiology of the two species (see original study plan for a summary of previous investigations with these two species). Also, as the population sizes of both species continue to increase in the Great Plains due to the large number of seedlings planted annually and from natural reproduction, we can expect the incidence of disease and insect problems to increase. Such an increase in disease and insect problems is already evident in the Great Plains region for these two species (M.O. Harrell, personal communication). Because J. virginiana and J. scopulorum are so dominant in the Great Plains region, and will probably continue to be until selections from suitable alternative species can be developed, it behooves us to know more about these two species; e.g.: to better understand the genetic control of important traits; how do the two species differ in response to environmental and biotic stresses (e.g., drought stress, diseases, insects); what are the genetic and physiological mechanisms associated with the stress characters; etc.

Based on the results of previous studies, Juniperus virginiana and J. scopulorum are believed to be highly introgressed (Flake et al. 1978; Van Haverbeke 1968; Comer et al. 1983). The native ranges of the two species include areas of sympatry and allopatry (Figure 1). Clinal variation patterns among the two species have been documented for morphological (Hall 1952; Van Haverbeke 1968), terpenoid (Flake et al. 1973; Flake et al. 1978); Adams 1983; Comer et al. 1983), and lipid (Van Haverbeke et al. 1968) characters, suggesting introgression. If the two species are truly introgressed, this will have implications in studying the genetics and physiology of the two species and selecting individuals better adapted to specific abiotic and biotic environments.

One character often used to differentiate the two species, and which should be investigated further, is the length of time required for cone and seed maturity. Mathews (1939) has determined that a single growing season is required for cone and seed maturation in Juniperus virginiana. While it is generally assumed that two growing seasons is required for cone and seed maturation in J. scopulorum, no direct study of the cone maturation cycle

of J. scopulorum has been completed. Fechner (1976) made anatomical observations during controlled interspecific crosses (one J. scopulorum tree as the female parent, crossed with one J. virginiana pollen parent). While the cones were removed from the J. scopulorum parent after only one growing season, the apparent lack of fertilization but normal female gametophyte development after one growing season in both wind-pollinated (presumably intraspecific) and control-pollinated (interspecific) cones indirectly confirms the two-year maturation cycle of J. scopulorum seeds. Therefore, we have continued to use cone maturity as the primary character to differentiate J. virginiana and J. scopulorum individuals and the associated open-pollinated progeny.

Several features of the chloroplast genome make it very well suited for studying plant phylogenetic and taxonomic relationships (Palmer and Zamir 1982). The base sequence of the chloroplast genome is sufficiently conserved within and between taxa that taxonomic comparisons are more discrete than for morphological or biochemical characters (Palmer and Zamir 1982; Palmer et al. 1985). Because of the relatively small size of the chloroplast genome--generally between 120 and 160 kb (Palmer 1985)--a reasonable number of resolvable fragments are produced in restriction endonuclease analyses of the chloroplast genome.

The conserved rate of evolution of the chloroplast genome, with its possible discreteness among taxa and populations, makes it especially useful for studying introgression in natural populations, provided that suitable markers can be found. The observed phenotypes in restriction enzyme analyses (the fragment length polymorphisms) are a direct expression of the genotype with no environmental masking. This is an advantage when intermediacy of environment results in intermediacy of morphological or biochemical characters, independent of gene exchange (on an evolutionary time scale), rather than "blending" due to introgressive hybridization. An assessment of introgression may also require the selection of nuclear markers (e.g., cone maturation requirements in Juniperus, isozymes, etc.), depending on the types of variability observed in the chloroplast DNA (cpDNA) of plant species or populations (e.g., whether or not species-specific probes are identified).

It is desirable to know the inheritance of the chloroplast genome in Juniperus if cpDNA variability is to be used to assess population genetic structure and possible introgression. The means of inheritance (maternal, paternal, or biparental) will govern the rate of spread and variability observed of cpDNA in a population or species. Inheritance of chloroplasts in gymnosperms has been shown to be paternal in every species or genus investigated to date (Neale et al. 1986, 1987; Ohba et al. 1971; Szmidt et al. 1987; Stine et al. 1987; Wagner et al. 1987), rather than the maternal or occasionally biparental inheritance of chloroplasts documented for most land plants (Sears 1980).

Controlled intra- and interspecific crosses provide the best test of whether two species are capable of hybridizing. The extent of interspecific crossability barriers, if any, relative to intraspecific crosses, and elucidation of the extent of genetic control of important crossability traits (e.g., seed germination, measures of filled but ungerminated seed, etc.) can be determined through controlled pollinations.

Chloroplast DNA analysis, in combination with control-pollinated intra- and interspecific crossability studies, will allow a more detailed assessment of the genetic structure of natural populations of Juniperus virginiana and J. scopulorum. This data can be used to determine if introgression is occurring in the two species, and thereby allow for better characterization of the genomes of the two species and aid in the selection of desirable genotypes and phenotypes.

RESEARCH OBJECTIVES

As stated in the original study plan (as submitted on November 4, 1987), the overall goals of this research project were to quantitatively and qualitatively determine the extent of chloroplast DNA (cpDNA) variability among provenances of Juniperus virginiana and J. scopulorum, and make some rough assessment of whether the two species are hybridizing in the field. The objectives of the research plan for the cooperative agreement were to: (1) carry out the controlled crosses necessary for the crossability and genetic control analyses (note: because the species require one or two growing seasons for cone maturation, the crossability and genetic control analyses were not part of this study); (2) determine the extent of cpDNA variability in natural populations of J. virginiana and J. scopulorum; and (3) construct a physical map of the chloroplast genomes of the two species.

MATERIALS AND METHODS

Controlled Crosses:

Controlled pollinations were carried out using trees in a progeny test plantation located at the Horning State Farm near Plattsmouth, Nebraska (41°N, 96°W). This plantation is part of the Great Plains Agricultural Council GP-13 regional seed source study of Juniperus virginiana and J. scopulorum (Van Haverbeke 1978). For the GP-13 study, open-pollinated cones were collected from 284 individual J. virginiana and J. scopulorum trees, identified on the basis of years required for cone maturity, from allopatric and sympatric areas in the Great Plains and Rocky Mountain region (Figure 2). Seedlings from 156 seedlots (132 seedlots of J. virginiana and 24 of J. scopulorum) were planted at the Horning State Farm in the spring of 1980 in four-tree plots with five replications. Six male and six female trees of each species were selected at the Horning Farm plantation on the basis of fecundity and provenance (origin of the female parent that produced the half-sib progeny tested in the Horning Farm plantation) to serve as parents in the controlled pollinations (Table 1). Only five J. scopulorum males of sufficient fecundity were found in the Horning Farm plantation, and therefore one additional J. scopulorum male was selected on the farm of W.T. Bagley, located east of Lincoln, NE. The origin of this individual is unknown, but was confirmed to be J. scopulorum on the basis of leaf morphology. The cpDNA genotypes of the parents will be determined later when the cpDNA of the full-sib progeny is analyzed.

A factorial mating design (North Carolina II) was used to carry out the controlled crosses (Figure 3). This design was selected over other mating designs because: (1) both J. virginiana and J. scopulorum are dioecious, precluding a strict diallel design; (2) the factorial design is efficient in the estimation of genetic parameters (additive and dominance variance components) if a large number of half-sib families are generated relative to full-sib families (Namkoong and Roberds 1974); and (3) the factorial design allows for a large number of parents to be sampled for a given number of total crosses. Each parent (male and female) was involved in six full-sib crosses; three intra- and three interspecific full-sib crosses per parent.

Branches bearing male strobili were collected from all of the male parents beginning on March 1, 1988, and thereafter on two to four day intervals, and brought to the lab for forcing. Pollen was forced by placing freshly cut ends of the branches in water in a heated room (approx. 28°C). J. virginiana and J. scopulorum branches were forced in different rooms, and branches from each tree were isolated in paper cones to prevent pollen contamination. Once the strobili on a given branch began to shed pollen, the strobili were removed from the branch by hand and ground in a mortar/pestle to release the pollen. The pollen was then placed in a vial, marked as to source and dates of collection and extraction, and placed in the refrigerator (4°C) until use. Because of the very small size of Juniperus pollen, and the resultant problems with static electricity, we could not get any of several

aspirator collection systems to work. The grinding did reduce pollen viability, based on pollen germination tests, but produced sufficient quantities of viable pollen. We are developing an aspirator or cellulose bag collection system for future crosses.

On March 18 and 21, 1988, branches on the female trees of both species were isolated using cellulose (sausage casing) bags. Two bags were used per cross, requiring a total of 12 isolation bags (branches) on each of the 12 females. In addition, one bag was placed on one female of each species to serve as unpollinated controls. Each bag on a given female was pollinated a minimum of two times, using an excess of pollen to insure pollination. Females were deemed receptive when the naked ovule(s) and its pollen drop could be observed in the open female strobilus. Not all strobili opened at once on a given branch; rather, female strobilus receptivity varied somewhat randomly from top to bottom of the tree, and from the branch tips inward, and therefore we made multiple pollinations of each bag. J. virginiana females were pollinated during the period March 28 through April 7, 1988, and J. scopulorum females were pollinated during the period April 11 through April 18, 1988. On average, there was almost a two week difference in female strobilus receptivity between the two species for the female parents in this study, and a similar difference in date of pollen shed among the male parents of the two species. Colored tape was placed on the branch at the base of the isolation bag to identify a given cross.

On June 6 and 7, 1988, the isolation bags were removed from the branches and insect exclusion bags were put on the branches. The insect bags were used at the request of Dr. Mary Ellen Dix, USFS RMF&RES-Lincoln, for a study of seed insects of J. virginiana and J. scopulorum, and were left on until the branches were cut to collect the cones. On October 4, 1988, all control-pollinated branches were cut from the J. virginiana female trees (each branch still in its insect exclusion bag) and brought to the laboratory to remove the cones. In the laboratory, the cones from each isolation bag were removed by hand, the number of full and aborted cones were counted and placed in separate paper envelopes, and the cross information recorded on each envelope. The cones are being stored at 4°C while Dr. Mary Ellen Dix collects data on the seed insects, after which time the cones will be stored at -20°C for three months before germination tests are initiated. This information was also recorded on data sheets for later crossability and germination analyses. The cones from the J. scopulorum female trees will be collected in October, 1989.

Variability of cpDNA:

Total DNA was extracted and purified from the leaves and fine branches of each of 12 Juniperus individuals using a modification of the CTAB procedure of Murray and Thompson (1980). The twelve Juniperus individuals represent a northwest to southeast transect, encompassing allopatric and sympatric areas of the range of the two species (Table 2). The purified DNA was then digested with the appropriate restriction enzyme (Table 3), fractionated in a 0.8% agarose gel (1X TBE buffer), and transferred to Zeta-Probe nylon membrane (BioRad) using the alkaline transfer procedure. Petunia cpDNA fragments cloned into pBR322 (from J. Palmer, U. Michigan; Figure 4; see Sytsma and Gottlieb 1986) were nick-translated with ^{32}P-labeled dCTP and hybridized to the membrane bound juniper DNA. Hybridization and prehybridization stringency conditions were 4X SSC, 0.5%SDS, 65°C. The membrane was then visualized by autoradiography using Kodak X-OMAT AR film in a -20°C freezer. After development, fragment sizes were determined by comparison to lambda/HindIII size standards.

To quantify the extent of cpDNA variability among the provenances of J. virginiana and J. scopulorum, the total fraction of shared fragments was calculated using the shared fragment method of Nei and Li (1979):

$$F = 2N_{xy} / (N_x + N_y) \ ,$$

where N_x and N_y are the number of fragments observed in individuals X and Y, respectively,

and N_{xy} is the number of fragments shared by individuals X and Y. Upholt's (1977) formula for the percentage of nucleotides separating a given pair of individuals (p) was used as the measure of genetic distance, and is calculated as:

$$p = 1 - \left[\frac{-F + (F^2 + 8F)^{1/2}}{2}\right]^{1/n}$$

where n is the number of base pairs recognized per cleavage site (n = 6 for all enzymes used in this study). The use of these formulas requires the following assumptions: (1) the observed fragment size differences are due only to base substitutions; (2) the distribution and frequency of cleavage sites in the Juniperus DNA are what would be expected in random sequences having the same base composition; and (3) fragments which are not homologous in sequence but have the same molecular weight are not scored as identical. Note that p will have large stochastic error when N_x, N_y, and N_{xy} are small (Nei and Li 1979). Therefore a large number of enzymes should be used and/or a large number of restriction fragments should be generated on average per individual. Nei and Li (1979) give a formula similar to that for p (δ_3), but it yields values almost identical to those for p (Lansman et al. 1981). Also, these formulae can be applied only in the comparison of individuals, or among populations or species with no intrapopulation or intraspecific polymorphisms. Nei and Li (1979) provide methods to account for this heterogeneity and an estimator of net nucleotide differences between two populations. However, it was applied only in analyzing the frequency of shared restriction sites between individuals, populations or species, rather than the frequency of shared fragments. Because of the above restrictions, comparisons will be made only among individuals.

Mapping of Juniperus cpDNA:

Mapping of the various Juniperus cpDNA restriction sites was carried out for one J. scopulorum individual (461-1-2), and this map used to determine equivalent maps for J. virginiana. An initial "rough" map was first developed by determining contiguous fragments in the J. scopulorum cpDNA using single digests, as indicated by hybridization to the various Petunia cpDNA probes for the different restriction enzymes. Double digests (digestion of the Juniperus total DNA using two restriction enzymes sequentially) were conducted to unambiguously map the J. scopulorum cpDNA restriction fragments.

Note: Because we also attempted to clone species-specific fragments of the J. virginiana and J. scopulorum cpDNA genomes (see next section) for further characterization, we have not yet completed the double digests needed for complete mapping. This will be discussed further in Results and Discussion.

Cloning of species-specific fragments:

When species-specific fragments of the J. virginiana and J. scopulorum genome were observed, it was decided to spend some time working out the protocols for cloning some of these fragments. Because we have not yet worked out the protocols necessary for isolating purified Juniperus cpDNA (vs. total DNA; a lot of ultracentrifuge time will be required, and we currently do not have access to an ultracentrifuge for such extended use, but rather only for intermittent use), it was decided to isolate the species-specific fragments from fractionated total DNA preparations with subsequent screening. This was accomplished by digesting the total DNA with the appropriate enzyme (depending on which species-specific fragment was desired), fractionation in a 0.8% agarose gel, and excision of appropriate segments of the gel when viewed on a UV light box. The DNA was electroeluted from the gel slice and purified according to the procedures of Maniatis et al. (1982). A sample of the purified electroeluted DNA was checked by agarose gel electrophoresis to insure it was of

the appropriate size. It was then ligated into the polylinker region of pUC18, transformed into competent DH5α E. coli cells (BRL) and recombinant colonies screened based on interruption of the β-galactosidase gene. Colonies having the appropriate Juniperus cpDNA fragments are then screened with the appropriate Petunia cpDNA probe using colony hybridization.

RESULTS AND DISCUSSION

Controlled Crosses:

In Table 4 the numbers of full cones from each of the full-sib controlled crosses for the J. virginiana females are listed. Again, because J. scopulorum requires two years for cone maturation, cones from the J. scopulorum females will not be collected until October, 1989. The cross results (Table 4) confirm that all pollen lots were viable and capable of fertilization. However, it is not yet known which if any of the bags within each of the crosses (not shown in Table 4--the number of cones in each bag were pooled) may show pollen contamination due to tears in the bags (which was recorded when observed in the field) or by some other means. The interspecific crosses with very high numbers of full cones (e.g., female J. virginiana 35 x male J. scopulorum 11, Table 4, etc.) are obviously suspect for pollen contamination. Pollen contamination will be assessed by comparing cpDNA genotypes of the parents vs. subsamples of the progeny. If the chloroplast DNA is inherited paternally in Juniperus, as has been documented in all other gymnosperms tested to date, then the cpDNA will be an excellent marker for pollen contamination. There is ample cpDNA variability within each species to assess pollen contamination in this wind-pollinated genus (see later). To our knowledge, these seed represent the only control-pollinated full-sib Juniperus seed and progeny available for study in the world. Fechner (1976) generated full-sib crosses of J. virginiana, but they were destroyed after germination due to the sampling scheme utilized. After the seed has been stored for three months at -20°C, germination tests will be conducted to assess intra- and interspecific crossabilities.

Juniperus cpDNA variability and mapping:

Table 5 lists the Petunia cpDNA probe / restriction enzyme combinations completed to date among the 12 Juniperus provenances listed in Table 2. The original supplier of the Petunia cpDNA clones (D. Neale, USFS, Berkeley, CA) did not have the *Pst* I-18 clone, and the *Sal* I-6 clone they originally sent us had previously undergone some type of rearrangement. We have just received (Jan. 9, 1989) a replacement for the *Sal* I-6 clone, and had to request the *Pst* I-18 clone from another source. These will be completed within the next month, along with the probe/enzyme combinations that need to be redone for accurate interpretation ("rd" in Table 5). Therefore, the comments made in the remainder of this section are pertinent to those probe/enzyme combinations tested, as shown in Table 5, and the inferences may change somewhat when the Juniperus cpDNA is probed with the *Pst* I-18 and *Sal* I-6 clones.

Of the eight restriction enzymes utilized in this study, restriction fragment length polymorphisms (RFLPs) were observed among the 12 Juniperus individuals (provenances) for all of the restriction enzymes except *Pvu* II. Until the mapping is completed, the exact nature of the mutations responsible for the observed variation cannot be unambiguously determined. However, the initial overlap maps (Figure 5) indicate most of the mutations among the 12 Juniperus individuals (provenances) are the result of insertions or deletions. No large rearrangements have been observed among the chloroplast genomes of the two Juniperus species (i.e., relative to each other), but the Juniperus chloroplast genome is highly rearranged relative to Petunia. In addition, a couple of the mutations in the Juniperus genome appear to have resulted in the loss of restriction sites (recognition sequences). The

resolution of mapping in this study is approximately 50 base pairs (bp). The majority of mutations in cpDNA are the result of small deletions and insertions on the order of 1 to 10 bp (Palmer 1985). Therefore, the fragment length polymorphisms observed in this study with Juniperus probably represent only a small portion of the actual total.

Single digest overlap maps show the Juniperus chloroplast genome to be highly rearranged relative to the Petunia chloroplast genome (Figure 5). Because of the taxonomic distance between the two groups, such differences are expected. However, the rearrangements observed in Juniperus are somewhat different than those observed in Petunia - radiata pine (Pinus radiata) comparisons (Strauss et al. 1988). In Juniperus, the cpDNA corresponding to most of the large single-copy region (P-16 through P-10 in Figure 5) shows little rearrangement relative to Petunia and radiata pine, except for a translocation in the region corresponding to the P-8 region of Petunia (Figure 5). Also, there is no indication that the Juniperus chloroplast genome contains the large inverted repeat common to most angiosperms, but absent in the two gymnosperms tested to date (radiata pine and Pseudotsuga menziesii, Strauss et al. 1988). No differentially sized fragments were observed among the Juniperus cpDNA genotypes when hybridized to the *Pst* I-1 and -4 clones. Asymmetry would be expected if the inverted repeats were present, as indicated by RFLPs associated with the small single copy region between them. Other rearrangements are suggested in the Juniperus data, but must await the completion of the double digests and gene mapping before they can be unambiguously assigned.

Table 6 lists some of the more common variation groupings observed for the 91 probe/enzyme combinations tested to date. When comparing Juniperus individuals that share common genotypes, the most common, and possibly ancestral, grouping is shown in Table 6: Juniperus individuals 1, 2, 4, 5, 7, and 11 are of cpDNA genotype 1, and individuals 3, 6, 8, 9, 10, and 12 are of cpDNA genotype 2. Each of the individuals also possess additional cpDNA variation beyond this basic pattern, due to either species-specific differences, possible heteroplasmy, other unique or less common forms of variation, or combinations of any of the previous forms of variation. Of the 12 Juniperus individuals, only individuals 9, 10, and 12 (as abbreviated in Table 6) possess the same cpDNA genotype for the enzymes tested to date; the remaining nine individuals possess unique cpDNA genotypes. Therefore, the Juniperus chloroplast genome appears to be quite diverse among populations and species (more later), and suitable for studies of population structure. The amount of variability present is not surprising given the outcrossing nature of this dioecious, wind-pollinated species group.

Given the results of previous studies which indicated J. virginiana and J. scopulorum to be highly introgressed (see Introduction for references), the number of species-specific RFLPs observed (Table 6) is very intriguing. The populations sampled include both allopatric and sympatric zones, and yet there is a very clear definition of species based on cpDNA variation. If the two species are hybridizing to the extent indicated by previous studies, then species-specific RFLPs should not be present. Rather, a mixing of the two groups should have been observed, at least among provenances in the sympatric zone. The two species were initially differentiated on the basis of years required for cone maturation (J. virginiana requires one growing season, and J. scopulorum requires two). Cone maturation is probably a nuclear encoded polygenic trait. Therefore the species-specific variation observed in the cpDNA must be due to a lack of natural hybridization and introgression between the two species, rather than linkage to cone maturation characters.

One individual, 751-1-1/1 (Table 2), shows evidence of possible heteroplasmy of the chloroplast genome (full data not shown). Heteroplasmy was recently reported in four Pinus trees from a sympatric population of Pinus banksiana and Pinus contorta (Govindaraja et al. 1988). This will also be investigated further in the future, as this Juniperus individual occurs in the sympatric zone of J. virginiana and J. scopulorum (Table 2).

Table 7 lists the total fraction of shared fragments (F) and the fraction of nucleotides separating (p) a given pair of individuals. While this data indicates generally good separation of the two species, the Juniperus individuals are also separated by the two basic

(possibly ancestral) groupings mentioned earlier (individuals 1, 2, 4, 5, 7, and 11 of cpDNA genotype 1, and individuals 3, 6, 8, 9, 10, and 12 of genotype 2; see Table 6), somewhat masking the species-specific differences. Also of interest is that the cpDNA of J. scopulorum individual 3 (581-1-3/1) shows greater similarity to J. virginiana individuals 6, 9, 10, and 12, than it does to the other J. scopulorum individuals (Table 7). The most divergent groupings are J. scopulorum individuals 1, 2, 4, and 5 versus J. virginiana individuals 9, 10, and 12. These data indicate that (1) the two species share common ancestry, but more recent crossability barriers separate them, or (2) crossability barriers do not exist, and the two species have only recently undergone natural hybridization and introgression. The results of the controlled crosses should help answer some of these questions.

Cloning of species-specific fragments:

To date only the *Eco* RI fragments corresponding to the P-12 and P-20 regions of the Petunia chloroplast genome have been cloned. However, none of the clones have been further characterized by screening, subcloning or sequencing. After the mapping experiments are completed, these clones will be further characterized, and additional species-specific fragments cloned. Provided the interspecific crosses show that interspecific hybridization is possible, these clones will then be used to investigate populations where interspecific hybridization may be occurring (i.e., used as homologous probes to screen Juniperus populations, rather than the heterologous Petunia probes).

SUMMARY

The large number of species-specific RFLPs observed in the cpDNA of J. virginiana and J. scopulorum indicate that the two species are not hybridizing to the extent postulated in previous studies. More detailed analysis of sympatric populations must be conducted before the existence and extent of localized interspecific hybridization can be determined. Analysis of the intra- and interspecific crosses made in this study will help greatly in determining whether the two species are capable of hybridizing. Among the parents in the controlled crosses in this study, the flowering phenologies of the two species differed by approximately two weeks, indicating flowering phenology could be at least a partial interspecific crossability barrier.

Single digest overlap maps indicate that the chloroplast genomes of the two Juniperus species do not contain any large rearrangements relative to one another, but are rearranged relative to results reported for other gymnosperms and angiosperms. The cloned species-specific Juniperus cpDNA fragments will be used as homologous probes in future studies to assess the extent of hybridization in specific sympatric populations. These clones will also be characterized more fully to determine the nature of the observed species differences in genome organization and function.

FUTURE WORK

The results from this study will be published in the following manner. After the double digests are completed, the mapping portion of the study will be submitted for publication in a refereed journal. The cpDNA inheritance analyses, to be completed after some of the full-sib progeny are grown as seedlings, will be published along with the provenance variation data compiled in this study, with S.G. Ernst and D.F. Van Haverbeke as coauthors. S.G. Ernst and D.F. Van Haverbeke will also submit to NSF a grant proposal to assess the extent of interspecific hybridization in sympatric populations, utilizing the species-specific cpDNA markers identified in this study.

Table 1. List of parents used to make the controlled pollinations, and location (point of origin) of its female parent.

Species	Sex	Accession Number[1] USFS	FFW	Location
J. scopulorum	Female	584-6-28/1	01	Reva, SD
		561-3-19/1	02	Larimore, ND
		581-3-11/3	03	Mosby, MT
		641-2-26/1	04	Chadron, NE
		582-6-20/1	05	Sarpy, MT
		641-1-25/2	06	Chadron, NE
	Male	561-1-28/3	07	Larimore, ND
		581-1-23/2	08	Mosby, MT
		641-1-11/2	09	Chadron, NE
		582-4-10/3	10	Custer, MT
		641-2-2/4	11	Chadron, NE
		----	12	Bagley Farm, Lincoln, NE
J. virginiana	Female	751-2-26/2	31	Endicott, NE
		1121-3-8/3	32	Kansas City, KS
		651-3-26/4	33	Valentine, NE
		752-2-21/2	34	Pollard, KS
		1122-2-26/3	35	Henrietta, OK
		711-5-3/3	36	St. Paul, NE
	Male	751-3-7/4	37	Fairbury, NE
		1121-2-4/1	38	Centerville, KS
		651-5-5/2	39	Mullen, NE
		752-4-12/1	40	Hillsboro, KS
		1122-4-5/1	41	Columbus, KS
		711-4-23/2	42	Gibbon, NE

[1]USFS accession numbers of the seed lots from which the trees listed originated, and include the seed zone of origin (first three digits), the female tree within that zone (next digit), and the location of the tree we worked with at the Horning Farm plantation (row/the tree number in the four-tree plot, where 1 = the tree closest to the southern terminus of the row). The FFW accession numbers include the female tree ID number and the pollen accession number. To get the full FFW accession number, add 5031-00XX to the J. scopulorum accession number and 5037-00XX to the J. virginiana accession number.

Table 2. List of Juniperus individuals included in the cpDNA variation study, including the location (point of origin) of its female parent.

Species Occupation Zone	Species	Accession Number[1] USFS	Location
Allopatric– J. scopulorum	J. scopulorum	461-1-6/2	Townsend, MT
	J. scopulorum	531-1-2/1	Sidney, MT
	J. scopulourm	581-1-3/1	Mosby, MT
	J. scopulorum	582-1-5/1	Billings, MT
Sympatric	J. scopulorum	641-1-7/2	Chadron, MT
	J. virginiana	651-2-6/4	Angora, NE
	J. virginiana	711-1-5/2	Grand Island, NE
	J. virginiana	751-1-1/1	Hastings, NE
Allopatric– J. virginiana	J. virginiana	1121-1-1/2	Kansas City, KS
	J. virginiana	1122-1-2/2	Henrietta, OK
	J. virginiana	1191-5-7/1	Clayton, OK
	J. virginiana	1332-1-7/1	Livingston, TX

[1]USFS accession numbers of the seed lots from which these trees originated, and include the seed zone of origin (first three digits), the female tree within that zone (next digit), and the location of the tree we worked with at the Horning Farm plantation (row/the tree number in the four-tree plot, where 1 = the tree closest to the southern terminus of the row).

Table 3. List of the restriction enzymes used in this study and its recognition sequence.

Restriction Enzyme	Recognition Sequence
Bam HI	G/GATCC
Eco RI	G/AATTC
Hind III	A/AGCTT
Kpn I	GGTAC/C
Pst I	CTGCA/G
Pvu II	CAG/CTG
Sal I	G/TCGAC
Xba I	T/CTAGA

Table 4. Numbers of fully developed cones for each of the intra- and interspecific controlled crosses, collected from the J. virginiana females in October, 1988.

J. virginiana Female Tree ID No.	Pollen Accession Number[1]												
	J. virginiana						J. scopulorum						Open-pollinated
	37	38	39	40	41	42	07	08	09	10	11	12	999
31	26	14	1				23	1				0	102
32		15	31	0			1	2	2				190
33			9	13	9			4	0	6			104
34				100	38	39			50	10	4		123
35	63				84	30				24	208	2	121
36	0	2				0	2				37	2	106

[1]The pollen accession numbers and female tree ID numbers are the same as those given in Table 1. Open-pollinated accession number is 9999, and is assumed to be J. virginiana pollen.

Table 5. A listing of the those Petunia cpDNA probe / restriction enzyme combinations which have been completed to date.[1]

Petunia cpDNA Probes[2]	Restriction Enzyme							
	Bam HI	*Eco* RI	*Hind* III	*Kpn* I	*Pst* I	*Pvu* II	*Sal* I	*Xba* I
Pst I - 1	X	*	X	X	X	X	X	X
- 3	X	X	X	X	X	X	X	X
- 4	X	*	*	X	X	X	X	X
- 6	X	X	X	X	X	X	X	*
- 8	X	*	*	X	X	X	X	X
-10	X	X	X	X	X	X	X	X
-12	X	*	X	X	X	rd	X	rd
-14	X	X	X	X	X	X	X	X
-16	X	rd(*)	rd	X	X	X	X	*
-18								
-19	X	*	X	X	X	rd	X	rd
-20	X	*	*	X	X	X	X	X
Sal I - 6								
- 8	X	X	*	X	X	X	X	X

[1]X = probe/enzyme screening completed; * = probe/enzyme screening completed, and species-specific genotypes were observed; "rd" = the probe/enzyme screening needs to be redone to assure accurate interpretation of the restriction fragment pattern; blank = probe/enzyme screening still to be completed.

[2]See Figure 4 for a map of the Petunia cpDNA cloned fragments.

Table 6. General groupings of cpDNA RFLPs observed among the 91 probe/enzyme combinations screened to date among the 12 Juniperus individuals.[1]

1) Monomorphic genotypes: observed among 60 of the probe/enzyme combinations.

2) Species-specific genotypes: 13 probe/enzyme combinations total (*Pst* I-16/*Eco* RI excluded).
   A) Only species-specific RFLPs observed (i.e., Juniperus individuals 1,2,3,4, and 5 are of one cpDNA genotype, and individuals 6,7,8,9,10,11, and 12 are another cpDNA genotype): 5 probe/enzyme combinations.
   B) Species-specific genotypes with additional cpDNA variability: 8 probe/enzyme combinations.

3) The most common grouping of variable cpDNA genotypes: 14 probe/enzyme combinations.
   Genotype 1: 1,2,4,5,7,11
   Genotype 2: 3,6,8,9,10,12

   A) Only genotype 1 vs. 2 differences observed: 4 probe/enzyme combinations.
   B) Genotype 1 vs. 2 differences observed with additional cpDNA variability: 10 probe/enzyme combinations (Note: for all 10 of these probe/enzyme combinations, individual no. 8 showed possible heteroplasmy).

4) Other cpDNA RFLPs (no common pattern): 4 probe/enzyme combinations.

[1]Juniperus individuals correspond to the following numbers (see Table 2 for the USFS accession number): 1 = 461-1-6/2, 2 = 531-1-2/1, 3 = 581-1-3/1, 4 = 582-1-5/1, 5 = 641-1-7/2, 6 = 651-2-6/4, 7 = 711-1-5/2, 8 = 751-1-1/1, 9 = 1121-1-1/2, 10 = 1122-1-2/2, 11 = 1191-5-7/1, 12 = 1332-1-7/1.

Table 7. Fraction of shared fragments (F: upper right portion of table) and fraction of nucleotides separating (p: lower left portion of table) a given pair of the Juniperus individuals. Individual designations (1, 2, 3, etc.) correspond to those given in Table 6.

	Individual											
	1	2	3	4	5	6	7	8	9	10	11	12
1		.9786	.8837	.9538	.9702	.8270	.9091	.8689	.8330	.8330	.9126	.8330
2	.0012		.9049	.9580	.9745	.8481	.9049	.8811	.8457	.8457	.9168	.8457
3	.0069	.0056		.8981	.8800	.9102	.8243	.8844	.9163	.9163	.8354	.9163
4	.0026	.0024	.0060		.9582	.8091	.9023	.8589	.8191	.8191	.9057	.8191
5	.0017	.0014	.0072	.0024		.8529	.9221	.8694	.8379	.8379	.9257	.8379
6	.0107	.0093	.0053	.0120	.0090		.8894	.9433	.9812	.9812	.9011	.9812
7	.0053	.0056	.0109	.0058	.0045	.0066		.9371	.9121	.9121	.9873	.9121
8	.0079	.0071	.0069	.0086	.0079	.0033	.0036		.9493	.9493	.9366	.9493
9	.0103	.0094	.0049	.0113	.0100	.0011	.0051	.0029		1.000	.9114	1.000
10	.0103	.0094	.0049	.0113	.0100	.0011	.0051	.0029	.0000		.9114	1.000
11	.0051	.0049	.0101	.0055	.0043	.0058	.0007	.0037	.0052	.0052		.9114
12	.0103	.0094	.0049	.0113	.0100	.0011	.0051	.0029	.0000	.0000	.0052	

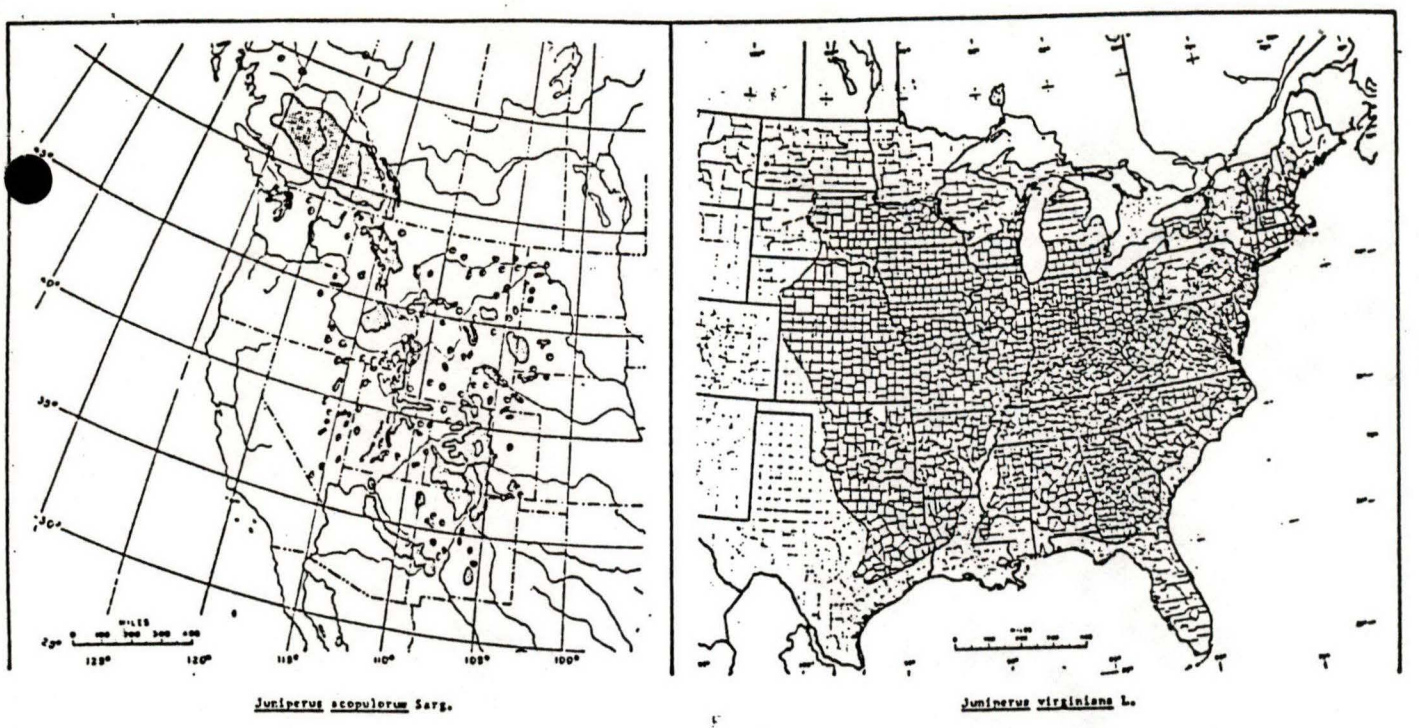

FIG. 1.—The botanical ranges of Rocky Mountain juniper (*Juniperus scopulorum* Sarg.) and eastern redcedar (*Juniperus virginiana* L.); after Fowells, 1965.

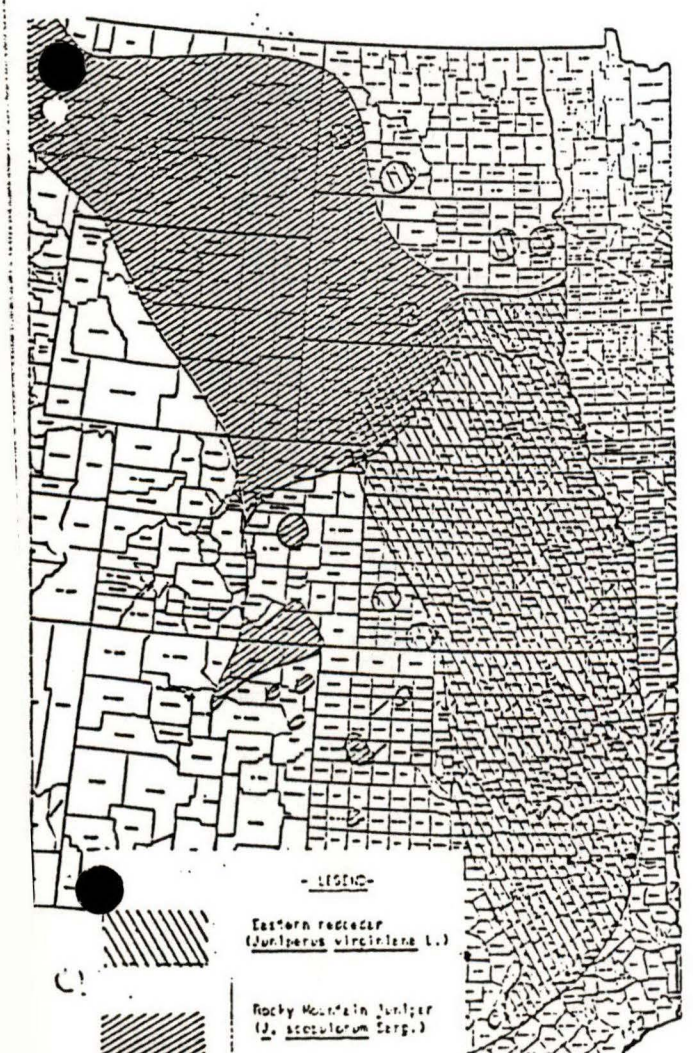

Figure 1. The botanical ranges of Rocky Mountain juniper and eastern redcedar, and an enlarged map of the zone of sympatry.

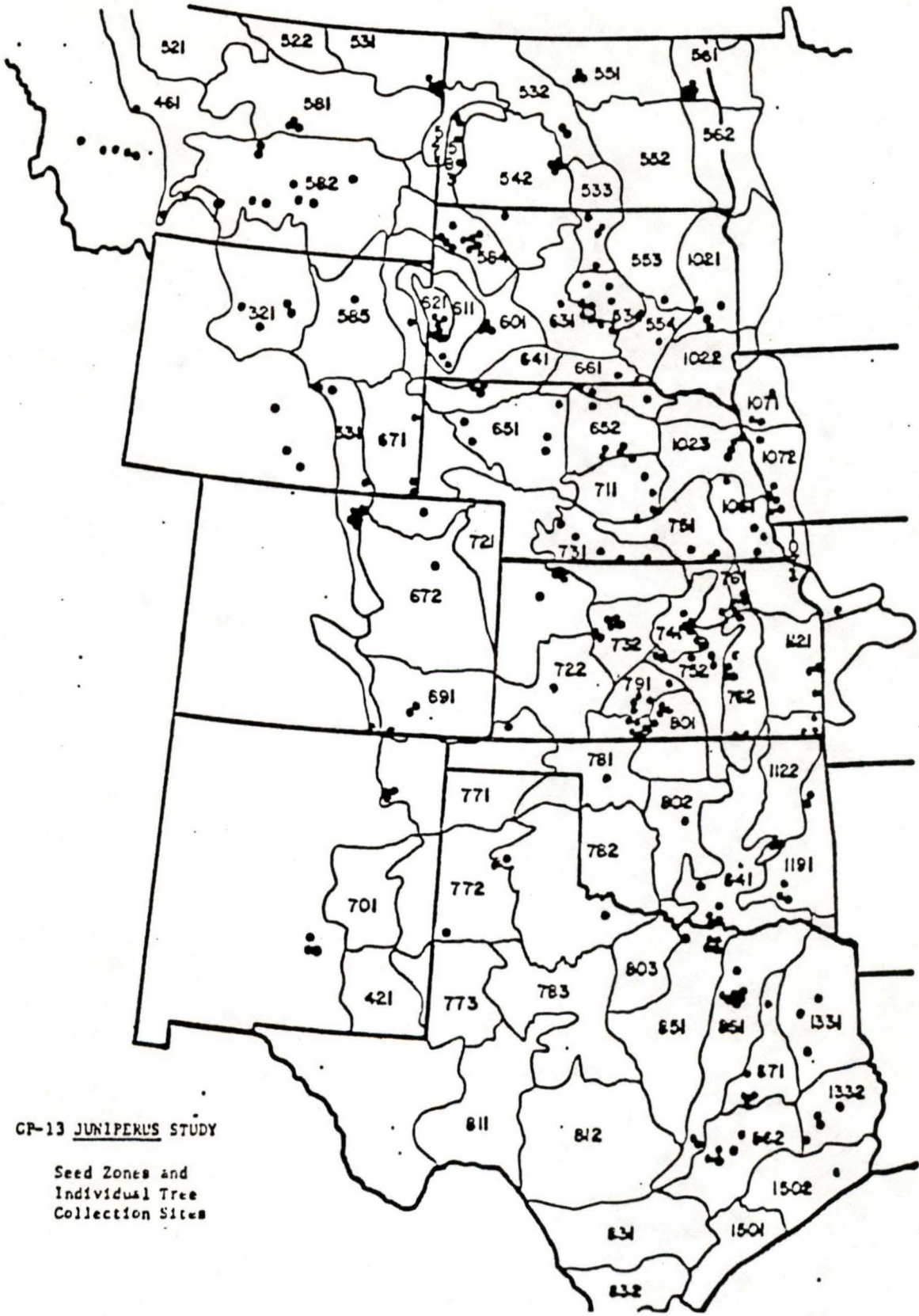

Figure 2. Individual tree collection sites and respective seed zones of the GP-13 Juniperus study.

		J. virginiana						J. scopulorum					
		13	14	15	16	17	18	19	20	21	22	23	24
J. v.	1	X				X	X	X				X	X
	2	X	X				X	X	X				X
	3	X	X	X				X	X	X			
	4		X	X	X				X	X	X		
	5			X	X	X				X	X	X	
	6				X	X	X				X	X	X
J. s.	7	X				X	X	X				X	X
	8	X	X				X	X	X				X
	9	X	X	X				X	X	X			
	10		X	X	X				X	X	X		
	11			X	X	X				X	X	X	
	12				X	X	X				X	X	X

-Crosses along the diagonal represent full-sib crosses between parents from the same or closely adjoining populations.

Figure 3. The layout of the factorial mating design used for the controlled pollinations among J. virginiana and J. scopulorum parents.

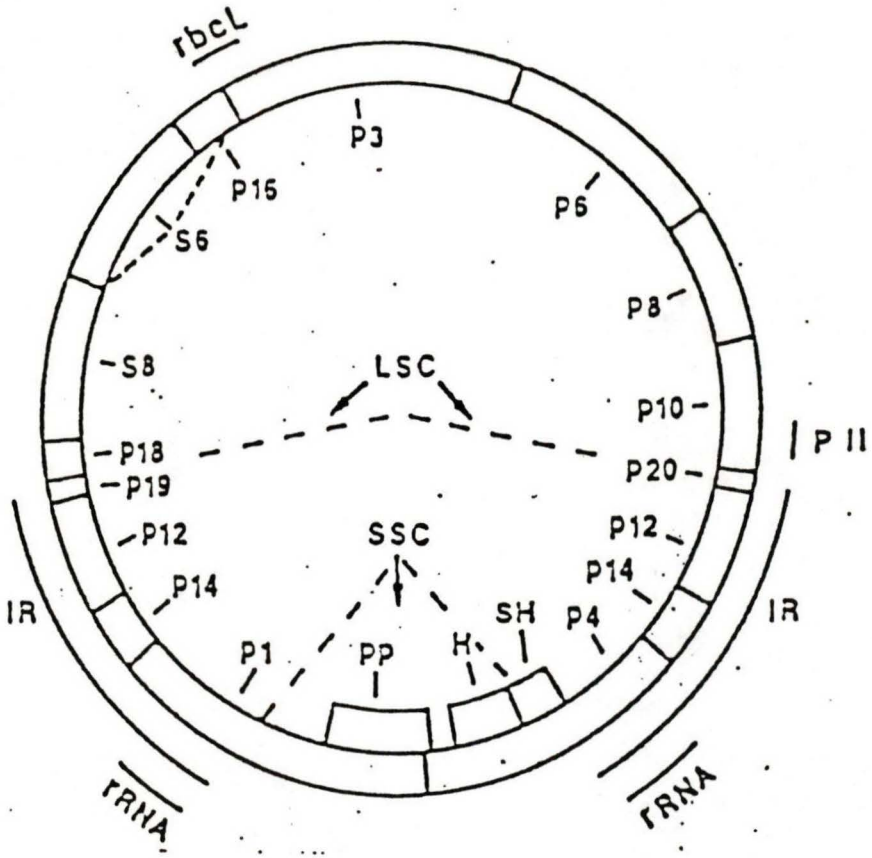

Figure 4. Restriction map of the petunia chloroplast genome, with the Pst I and Sal I cloned fragments (cloned into pBR322 by J. Palmer, U. Michigan) shown as P-n and S-n, respectively, which are being used as heterologous probes to analyze RFLP's in Juniperus. The restriction map has been copied from Sytsma and Gottlieb (1986).

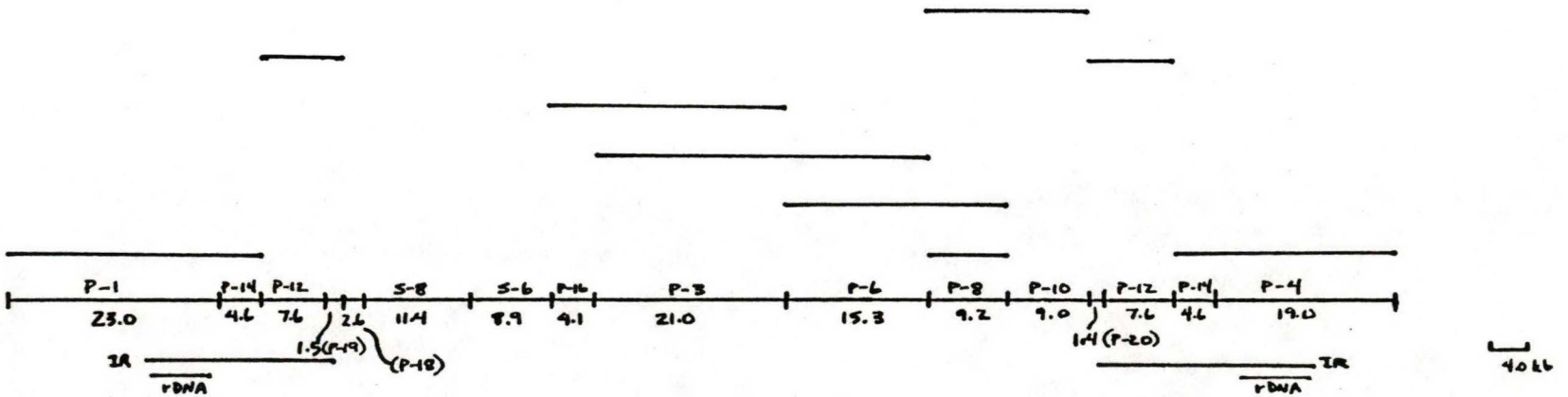

Figure 5. Single digest overlap map of the Juniperus chloroplast genome (upper series of discontinuous lines) relative to the Petunia chloroplast genome (lower map, showing the Pst I and Sal I restriction sites--see also Figure 4). The Juniperus cpDNA lines show regions of similarity to Petunia and which are contiguous or colinear in Juniperus.

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January 16, 1989

Dr. David F. Van Haverbeke
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East Campus, UNL

Dear Dave:

Enclosed please find two copies of the final report for Cooperative Agreement No. 28-C7-438, "Chloroplast DNA Variability and Population Genetic Structure of Juniperus virginiana and J. scopulorum". Please let me know if there are any questions or comments about the final report that would require my input.

I would like to thank you and the Rocky Mountain Forest and Range Experiment Station for providing the funds to do this study. Thanks also for allowing me to work with the GP-13 Juniperus progeny, and for sharing of your expertise during several phases of this study. Compared to results reported previously, the cpDNA study has revealed a very different picture regarding the extent of possible introgression between J. virginiana and J. scopulorum, and should provide an excellent foundation for future research efforts and the ability to compete for grant monies.

Sincerely,

Stephen G. Ernst
Assistant Professor

Enclosure

xc: Dr. Gary Hergenrader